Biochemical and Biophysical Research Communications 422 (2012) 59–63

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Over-expression in *Escherichia coli*, purification and reconstitution in liposomes of the third member of the OCTN sub-family: The mouse carnitine transporter OCTN3

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ARTICLE INFO

Article history:
Received 19 April 2012
Available online 27 April 2012

Keywords:
OCTN3
Expression
Purification
Transport
Reconstitution
Liposomes

ABSTRACT

pET-21a(+)-mOCTN3-6His was constructed and used for over-expression in *Escherichia* coli Rosetta(DE3)-pLysS. After IPTG induction a protein with apparent molecular mass of 53 kDa was collected in the insoluble fraction of the cell lysate and purified by Ni^{2+}-chelating chromatography with a yield of 2 mg/l of cell culture. The over-expressed protein was identified with mOCTN3 by anti-His antibody and reconstitution in liposomes. mOCTN3 required peculiar conditions for optimal expression and reconstitution in liposomes. The protein catalyzed a time dependent [^{3}H]carnitine uptake which was stimulated by intraliposomal ATP and nearly independent of the pH. The K_m for carnitine was 36 μM. [^{3}H]carnitine transport was inhibited by carnitine analogues and some Cys and NH_2 reagents. This paper represents the first outcome in over-expressing, in active form, the third member of the OCTN sub-family, mOCTN3, in *E. coli*.

1. Introduction

Membrane transport systems allow uptake and efflux of a very large variety of molecules such as nutrients, catabolites and cofactors or their precursors [1–5]. Genes coding membrane transporters, indeed, represent a significant fraction of the genome of all organisms [6]. The occurrence of severe pathologies caused by genetic defects demonstrates the central role of these proteins in maintaining cell homeostasis [5,7]. In spite of their relevance, functional and structural studies on higher animal membrane transporters only recently have largely spread out. The study of these proteins, is challenged by the difficulties in expressing them in bacterial hosts and, hence, in purifying and studying functional properties in *in vitro* experimental systems [8]. Recently, over-expression in *Escherichia coli* of the human OCTN1 and OCTN2 transporters [9,10] was obtained. The hOCTN1 recombinant protein was then reconstituted in liposomes and functionally characterized [11,12]. The two transporters belong to a small protein sub-family, OCTN (Organic Cation Transporters Novel), to which a third member, OCTN3 also belongs. The coding genes for OCTN's have been identified only in higher mammals. Differently from OCTN1 and 2, the gene of OCTN3 has not yet been annotated in *Homo sapiens* genome [13,14], even though, the OCTN3 protein has been detected in human skin and sperm using antibody against mouse (m)OCTN3 [15–18]. The functional information concerning the OCTN3 transporter comes from experiments performed with murine cells [16,19–21] or in cell lines in which the transporter was artificially expressed [13]. The data obtained in the cell systems show that the OCTN3 is a carnitine transporter [19–21] and that it also recognizes acetylcarnitine [16]. In the present work the over-expression of mOCTN3 and its reconstitution in liposomes was obtained.

2. Materials and methods

2.1. Materials

E. coli Rosetta(DE3) and pET-21a(+) were purchased from Novagen; full length cDNA (IRCLp5011H1113D) from Source BioScience UK Limited; cloning reagents from Fermentas; yeast extract from Applichem; Triptone, Amberlite XAD-4, 3-sn-phosphatidylcoline from egg yolk, Triton-X100, Sephadex G-75, His-select Ni-affinity gel, protease inhibitor cocktail and L-carnitine from Sigma; L-[*N*-methyl-3H]carnitine hydrochloride from PerkinElmer. All the other reagents were of analytical grade.

Abbreviations: TEA, tetraethylammonium; PLP, pyridoxal 5-phosphate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate; *p*-OHMB, *p*-hydroxymercuribenzoate; GABA, γ-aminobutyric acid; ANTP, adenosine 5′-(β,γ-imido)triphosphate.

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http://dx.doi.org/10.1016/j.bbrc.2012.04.105

2.2. Cloning of mOCTN3

The 1692 bp cDNA coding for mOCTN3 (SLC22A21) was amplified from the full length cDNA with the forward 5′-GGGAATTCCATATGCTTGACTACGACGAGGTGA-3′ and reverse 5′-CCGCTCGAGAAAGCCTTTAGGTTCGGAGGT-3′, containing the *NdeI* and *XhoI* sites, respectively. The cDNA was then cloned in pET-21a(+). The recombinant pET-21a(+)-mOCTN3, carries the extra C-terminal sequence LEHHHHHH.

2.3. Expression of recombinant mOCTN3 protein in E. coli

E. coli Rosetta(DE3) strain was transformed with the pET-21a(+)-mOCTN3 by calcium chloride treatment. Selection of colonies was performed with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. *E. coli* Rosetta(DE3) positive cells were inoculated in 100 mL of TY 2X medium (1.6% Tryptone, 1% Yeast extract, 0.5% NaCl, pH 7.0) supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol, and cultured overnight at 37 °C. 50 mL of cell culture was transferred to 0.5 L of TY 2X medium supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. Expression was induced by 0.1 mM IPTG for 6 h at 28 °C. Bacteria were centrifuged at 3000*g* for 10 min at 4 °C. The bacterial pellet (about 3 g wet weight) was resuspended in 30 mL start buffer (500 mM NaCl, 40 mM Hepes-Na, pH 7.4) plus 0.2 mL of protease inhibitor cocktail and 0.5 mM PMSF. Cells were disrupted by mild sonication at 4 °C (10 min in pulses of 1 s sonication, 1 s intermission). The soluble and the insoluble cell fractions were separated by centrifugation at 20,000*g* for 30 min at 4 °C. Proteins patterns were analyzed by SDS–PAGE.

2.4. Purification of recombinant mOCTN3-6His protein

The insoluble fraction was washed with 0.1 M Tris/HCl pH 8.0, then solubilized by 10 mM 1,4-dithioerythritol (DTE), 3 M urea, 0.8% sarkosyl, 200 mM NaCl, 10% glycerol, 20 mM Tris/HCl pH 8.0, and centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was applied onto a His-select Ni-chelating affinity column (0.5 cm diameter × 3 cm height) pre-conditioned with 8 ml of 0.1% sarkosyl, 200 mM NaCl, 10% glycerol, and 20 mM Tris/HCl pH 8.0. The elution was performed with 5 ml of a buffer containing 0.1% Triton X-100, 200 mM NaCl, 10% glycerol, 5 mM DTE, 20 mM Tris/HCl pH 8.0 (washing buffer), 3 ml of the same buffer containing 10 mM imidazole and then 2 ml of a buffer containing 0.1% Triton X-100, 10% glycerol, 5 mM DTE, 20 mM Tris/HCl pH 8.0, 50 mM imidazole. Fractions of 1 ml were collected. The purified protein was eluted in the tenth fraction.

2.5. Reconstitution of the mOCTN3 transporter into liposomes

The purified mOCTN3 was reconstituted by removing the detergent from an initial mixture of 6 μg purified protein, 70 μl of 10% Triton X-100, 120 μl of 10% egg yolk phospholipids in the form of sonicated liposomes, 10 mM ATP, 10 mM Tris/HCl pH 8.0 in a final volume of 700 μl by Amberlite XAD-4 in a batch-wise procedure [11,12].

2.6. Transport measurements

Proteoliposomes (550 μl) were passed through a Sephadex G-75 column (0.7 cm diameter × 15 cm height) preequilibrated with 10 mM Tris/HCl (pH 8.0) and divided in samples of 100 μl. Transport was started by adding 0.1 mM [^{3}H]-carnitine to the samples and stopped by passing the samples through Sephadex G-75 columns (0.6 cm diameter × 8 cm height) to separate the external from the internal radioactivity. Liposomes were eluted in 2 min at 4 °C to avoid substrate leakage, with 1 ml 50 mM NaCl and collected in 4 ml of scintillation mixture, vortexed and counted. The experimental values were corrected by subtracting the respective control (without incorporated protein). The amount of radioactivity taken up by liposomes without protein was always lower than 15% of that found in proteoliposomes. Experimental data were fitted using the Grafit software (version 5.0.13).

2.7. Other assays

Proteins were separated by 12% SDS–PAGE, performed according to Laemmli [22]. Quantitative evaluation of Coomassie-stained protein bands was carried out using the Chemidoc imaging system equipped with Quantity One software (Bio-Rad).

3. Results

3.1. Expression of the mOCTN3 construct

The sequence of the full length mOCTN3 cDNA corresponded to the deposited GeneBank NM_019723 cDNA (not shown). The cDNA was cloned in the pET-21a(+) vector carrying a C-terminal 6His tag and the pET-21a(+)-mOCTN3-6His transfected in Rosetta(DE3)-pLysS. Firstly, LB and TY 2X media were used for cell growth; slightly better expression was obtained with TY 2X. In this condition, the presence of an expression product analyzed on SDS–PAGE, was observed in the insoluble fraction of the cell lysate which was absent in the soluble fraction and in the non-induced cell lysate. More abundant expression was obtained at 28 °C respect to 37 °C (not shown). The best condition was obtained at lowest IPTG concentration (0.1 mM; Fig. 1A line 2) with an amount of protein which was about three times respect to 1 mM IPTG (Fig. 1A line 4), even though the protein migrated still as a diffused band.

3.2. Purification of the expressed mOCTN3

Since it was not soluble in non-ionic detergents, expressed mOCTN3 was solubilized with 0.8% sarkosyl (Fig. 1B lane 1) and loaded onto a Ni^{2+}-chelating column. Virtually all bacterial proteins were removed by the washing buffer (not shown). mOCTN3 was then eluted with 50 mM imidazole; it resulted as a single protein band on SDS–PAGE (Fig. 1B, lane 2). The mOCTN3 in the purified fraction was enriched about 10 folds with respect to the cell lysate, as calculated on the basis of quantitative evaluation of the protein fractions (Fig. 1B and see the related legend). The mOCTN3 in the cell lysate and in the purified fraction was detected by an anti-His antibody with an apparent molecular mass of 53 kDa (Fig. 1C). In the purified fraction the protein band was sharper than in the extracted cell lysate. A yield of 1.5–2.0 mg/l of cell culture (6 g wet weight) was obtained in the different preparations.

3.3. Functional properties of the reconstituted mOCTN3

The mOCTN3 after purification was soluble in 0.1% Triton X-100. The reconstitution in liposomes was optimized. Best conditions were obtained with 6 μg/ml protein and a detergent/lipid (w/w) ratio of 0.6 (not shown).

The time course of [^{3}H]carnitine uptake was analysed in proteoliposomes containing ATP. As shown by Fig. 2, the specific transport in the presence of intraliposomal carnitine was nearly coincident with that in the absence of internal substrate, indicating a uniport transport mode. Fig. 2 shows also that the transport activity in the absence of intraliposomal ATP was much slower than in its presence. Intraliposomal ANTP, the non-hydrolysable analogue, exerted the same effect of ATP; while, externally added

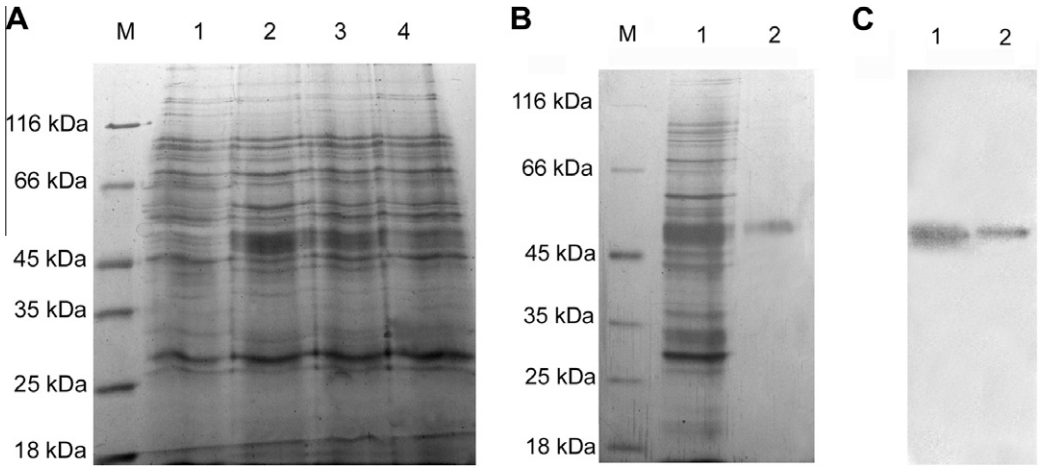

Fig. 1. Expression and purification of the mOCTN3 protein. Proteins were separated by SDS–PAGE and stained as described in Materials and methods. (A) Lane M: molecular mass markers; lane 1, uninduced cell lysate; lanes 2–4: insoluble fraction of cell lysate obtained in presence of 0.1 mM (lane 2), 0.4 mM (lane 3), 1 mM (lane 4) IPTG respectively (40 μg). (B) Lane 1, sarkosyl solubilised cell lysate (30 μg); lane 2, purified mOCTN3 (3 μg). (C) Immunoblotting of the mOCTN3 purification: lanes 1 and 2, 3,3′-diaminobenzidine immunodetection by anti-His antiserum (1:1000) of PVDF membrane after Western blotting of the same protein fractions of lanes 1–2 of (B).

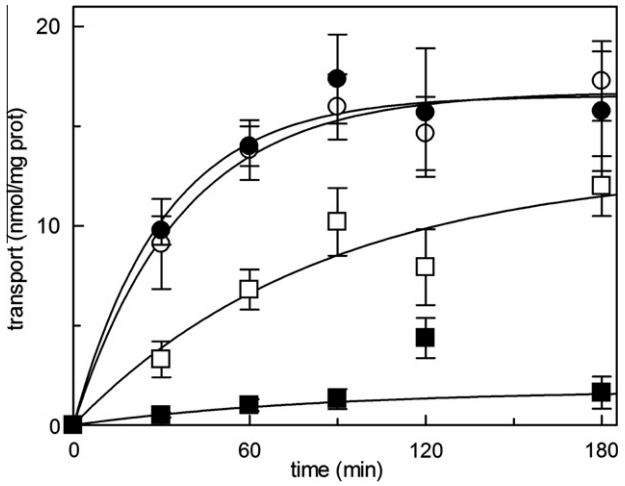

Fig. 2. Time course of carnitine uptake by reconstituted proteoliposomes. The reconstitution was performed as described in Materials and methods. Transport was started adding 0.1 mM [^{3}H]-carnitine at time zero to proteoliposomes (○, ●, □) or proteoliposomes reconstituted with protein treated for 20 min at 100 °C (■). No substrate (○) or 10 mM carnitine (●) was present inside the proteoliposomes. In (□) the protein has been reconstituted without intraliposomal ATP. The transport reaction was stopped at the indicated times by directly passing the proteoliposomes through Sephadex G-75 columns as described in Materials and methods. The values are means ± S.D. from three experiments.

ATP did not stimulate transport (not shown). The data of time courses were fitted in a first order rate equation from which the initial rate was derived as the product of k (the first order rate constant) and the transport at the equilibrium. Initial rates of 0.50 ± 0.069, 0.54 ± 0.097 or 0.15 ± 0.060 nmol/min/mg protein were obtained in the absence, in the presence of internal carnitine or in the absence of ATP, respectively. Transport in liposomes reconstituted with boiled mOCTN3 protein was much lower than that measured with native protein, indicating that the transport was protein-mediated. Transport activity only slightly increased by increasing the pH from 6.5 to 8.5 (not shown), thus being nearly unaffected by pH in line with previous reports [13,20,21]. To test the specificity of the reconstituted transporter towards hypothetical substrates, a number of molecules with structural homology with carnitine were added together with labelled carnitine to the proteoliposomes and the residual transport activity measured in comparison with the control, i.e., without additions. It has to be highlighted that palmitoylcarnitine has been used at 50 μM concentration because of its detergent property. GABA and acetylcarnitine inhibited the transporter by more than 50%; TEA, acetylcholine, butyrobetaine, butyric acid and palmitoylcarnitine inhibited about 40%, while ergothioneine, choline, creatine or creatinine had nearly no effect at the tested concentration (Fig. 3A). The SH reagents $HgCl_2$, mersalyl and pOH-mercury benzoate inhibited the transport by more than 50% at the higher concentrations tested (Fig. 3B). While the hydrophilic SH reagent MTSET and the NH_2 reagent pyridoxal phosphate had a lower inhibitory effect. The inhibition was always concentration dependent.

Kinetics of mOCTN3 were analysed as dependence of the transport rate on the substrate concentration. The experimental data were interpolated by the Michaelis–Menten equation (Fig. 4) from which half-saturation constant (K_m) of 36 ± 9.1 μM and V_{max} of 1.5 ± 0.18 nmol/min per mg protein were derived.

4. Discussion

Few examples of over-expression in bacteria and reconstitution of mammalian plasma membrane transporters are available so far [9–12,23–25]. This is mainly due to the low compatibility of mammalian mRNAs and proteins with bacterial translation and folding systems. Notwithstanding, the bacterial expression remains the most useful tool for obtaining large scale production of purified proteins for structural studies and the methodology of reconstitution in liposomes is the most up-to-date for obtaining reliable information on the function of membrane transporters and on interactions with exogenous molecules such as pharmacological and toxic compounds. Strategies for large scale expression of the hOCTN1 and hOCTN2 transporters have been recently pointed out [9,10]. However, the third member of the OCTN sub-family was still missing. To obtain its expression, several conditions had to be modified respect to the procedures used for the first two members: expression vector, bacterial cells and growth conditions. In particular and differently from the other OCTN proteins [9,10], the concentration of IPTG had to be drastically decreased to obtain a significant expression of mOCTN3. The expressed protein extracted from the insoluble fraction of cell lysate appeared as a diffused protein band on SDS–PAGE. This could be due to the insolubility and to the presence of different oxidation states of the extracted protein, which leads to a non-homogeneous run even under the reducing condition of the

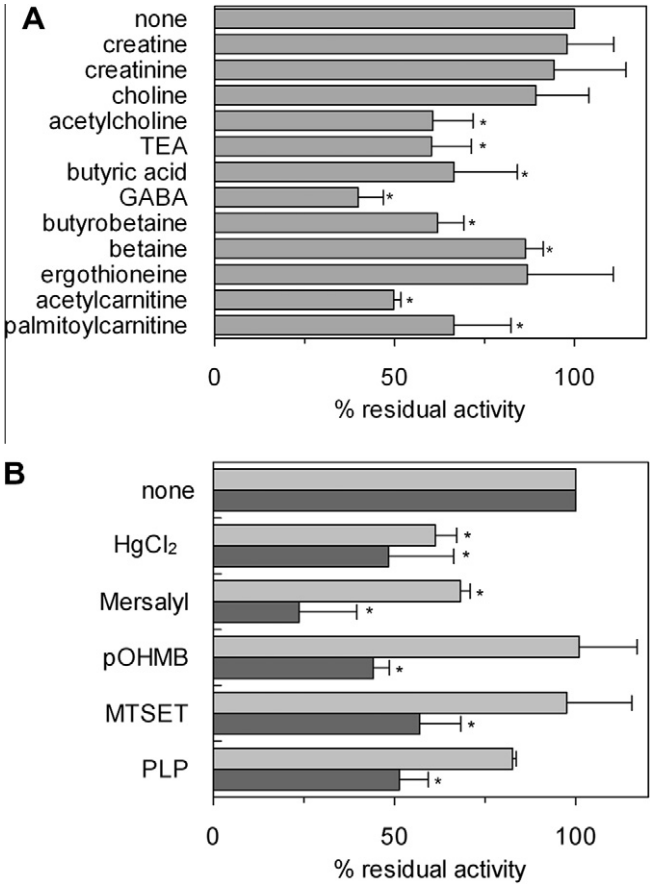

Fig. 3. Effect of different substrates and inhibitors on the reconstituted mOCTN3. Transport was measured as 0.1 mM [^{3}H]-carnitine uptake into proteoliposomes, reconstituted as described in Materials and methods, in 30 min. (A) The molecules were added 1 min before the labelled substrate. The concentration used was 1 mM except for palmitoylcarnitine (50 μM). Percent residual activity was calculated for each experiment with respect to the control sample (referred as 100%). (B) The inhibitors were added 1 min before the labelled substrate. The concentration used for $HgCl_2$and pOHMB was 0.02 mM (light grey) and 0.2 mM (dark grey), for mersalyl and MTSET was 0.1 mM (light grey) and 1 mM (dark grey), for PLP was 1 mM (light grey) and 2 mM (dark grey). Percent residual activity was calculated for each experiment with respect to the control sample (referred as 100%). The results are means ± S.D. of the percentage of three experiments. (*) Are significantly different from the control as determined by Student's *t*-test ($p < 0.05$).

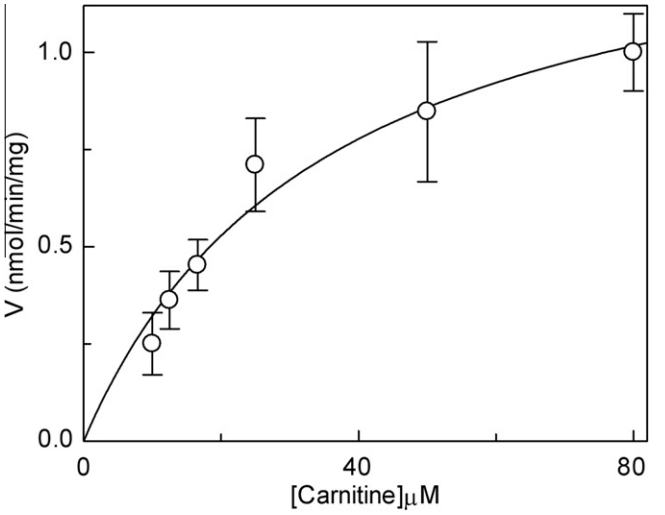

Fig. 4. Kinetic analysis of the reconstituted mOCTN3. Transport rate was measured adding [^{3}H]-carnitine at the indicated concentrations to proteoliposomes, reconstituted as described in Materials and methods, in 20 min, i.e., in within the initial linear part of the time course (see Fig. 2). Results were plotted according to Michaelis–Menten as transport rate vs. carnitine concentration. The results are means ± S.D. from three experiments.

electrophoresis; this is in line with the presence of seven Cys residues in the transport protein and with its sensitivity to SH reagents (see below). The purification procedure allows removal of the ionic detergent sarkosyl used for solubilizing the expressed protein and its substitution with the non-ionic Triton X-100. This process leads to refolding of the mOCTN3 which, then, appeared as a sharper band. As for the expression, the parameters for reconstitution of the transporter in liposomal membrane differed from those found to be optimal for hOCTN1 [11], in spite of the high similarity (83%) between the two proteins. The proteoliposome tool, which allows transport assay in absence of interferences, may reveal particularly useful in case of mOCTN3 since the simultaneous presence of the carnitine transporter OCTN2 in cells, could lead to interferences in measuring functional parameters. For a reliable characterization of the reconstituted protein, it has been firstly verified whether some basic functional features of mOCTN3 in proteoliposomes corresponded to those described in cell systems. Carnitine transport catalysed by the recombinant mOCTN3 (Fig. 2) was about one order of magnitude higher than that catalysed by hOCTN1 [11] and occurred by a uniport mode according to data described in cells [13]. The K_m of mOCTN3 for carnitine in proteoliposomes was similar to that measured in cell systems [15,16]. These data indicated that the reconstituted mOCTN3 was properly refolded. Furthermore, the proteoliposome tool allowed to obtain additional information on the function of the transporter. Intraliposomal, not extraliposomal, ATP stimulates the mOCTN3 transport activity. This finding indicates that the transport protein, being regulated by ATP, is inserted in the liposomal membrane with the same orientation of cell membrane, as described for hOCTN1 [11,12] and rat kidney OCTN2 [26]. Specific inhibitors for SH and NH_2 amino acid residues inhibited the transporter suggesting an important role of Cys and Lys residues in the protein function. mOCTN3 is inhibited by acetylcarnitine in agreement with previous data in cells showing this molecule as a substrate of mOCTN3 [16]. In proteoliposomes, it has been found that palmitoylcarnitine at 50 μM concentration inhibited mOCTN3, indicating that this hypothetical substrate may have high affinity for the transporter. Also the physiological compounds GABA, acetylcholine and butyrobetaine efficiently inhibited the reconstituted transporter suggesting possible roles of OCTN3 in nervous tissues and in the non-neuronal cholinergic system which is active in several districts such as intestine [27]. Accordingly, mOCTN3 which is expressed at high level in testis, is also expressed, even though at a lower level, in nervous tissue, brain blood barrier, intestine, kidney [13,28]. Indeed, GABA inhibited carnitine accumulation was firstly reported both in brain endothelial cells and in neurons but the involved transporter network was not fully understood [29]. Later on, OCTN3 expression has been shown in adult murine brain [19,30]. Taken together, these findings substantiate a role of mOCTN3 in carnitine homeostasis maintenance in brain. Data here reported are also consistent with the function of transporting carnitine and acylcarnitines in testis for spermatozoa maturation [15,16] and in absorption of carnitine through epithelia [20,21,30]. Moreover, results here described may also be in line with the proposed role for OCTN3 in carnitine and acylcarnitine trafficking through peroxisomal membranes [17]. The functional data on the mouse protein allows to hypothesize that the human isoform, whose existence has been suggested [15,17,18], should play similar functions, as it has been described for OCTN1 [13]. Indeed, OCTN3 could be one of the players of the carnitine transporter network [31].

In our knowledge, this work represents the first outcome in over-expressing functional mOCTN3 transporter. The basic properties

found in the *in vitro* experimental system allow to conclude that, as in cells, it is specific for carnitine. Indeed, the study of the mouse transporter represents, thus far, the only available tool for studying the third member of the OCTN sub-family. These and further studies on mOCTN3 may be relevant to gain insights in the probable involvement of hOCTN3 in IBD, celiac and Alzheimer's diseases, as suggested in some previous reports [18,21,30].

Acknowledgment

This work was supported by funds from Programma Operativo Nazionale 01_00937 "Modelli sperimentali biotecnologici integrati per lo sviluppo e la selezione di molecole di interesse per la salute dell'uomo" – MIUR and from University of Calabria (ex 60% 2009–2010).

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